Circadian and Light-Induced Transcription of Clock Gene *Per1* Depends on Histone Acetylation and Deacetylation

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Circadian clock genes are regulated through a transcriptional-translational feedback loop. Alterations of the chromatin structure by histone acetyltransferases and histone deacetylases (HDACs) are commonly implicated in the regulation of gene transcription. However, little is known about the transcriptional regulation of mammalian clock genes by chromatin modification. Here, we show that the state of acetylated histones fluctuated in parallel with the rhythm of mouse *Per1* (*mPer1*) or *mPer2* expression in fibroblast cells and liver. Mouse CRY1 (mCRY1) repressed transcription with HDACs and mSin3B, which was relieved by the HDAC inhibitor trichostatin A (TSA). In turn, TSA induced endogenous *mPer1* expression as well as the acetylation of histones H3 and H4, which interacted with the *mPer1* promoter region in fibroblast cells. Moreover, a light pulse stimulated rapid histone acetylation associated with the promoters of *mPer1* or *mPer2* in the suprachiasmatic nucleus (SCN) and the binding of phospho-CREB in the CRE of *mPer1*. We also showed that TSA administration into the lateral ventricle induced *mPer1* and *mPer2* expression in the SCN. Taken together, these data indicate that the rhythmic transcription and light induction of clock genes are regulated by histone acetylation and deacetylation.

Most organisms have physiological and behavioral rhythms, so-called circadian rhythms, having an intrinsic period of approximately 24 h. The circadian clock is an endogenous oscillator that controls daily physiological and behavioral rhythms. In mammals, molecular oscillators exist in the suprachiasmatic nucleus (SCN) of the brain, a master clock (19, 21, 31), and also in peripheral tissues (24, 48). Even in fibroblast cell lines, clock genes are induced rhythmically under certain conditions (1, 5, 47). The core circadian system consists of an interacting transcriptional-translational feedback loop of clock genes in an individual cell (11, 31). A negative feedback loop involves the regulation of two period genes (*Per1* and *-2*) and two cryptochrome genes (*Cry1* and *-2*) (22, 33). The rhythmic transcription is driven by the basic helix-loop-helix–PAS protein (CLOCK-BMAL1) complex, which binds the E-box on the *mPer1* and *mPer2* genes (14). This CLOCK-BMAL1-mediated transcription is, in turn, repressed by the translated products of clock genes, such as the mPER and mCRY protein complex, which translocate to the nucleus (14, 17, 22, 33).

On the other hand, rapid inductions of *mPer1* and *mPer2* are also involved in phase resetting of the circadian rhythm (3, 4, 34). A light pulse during subjective night induced rapid increases in *mPer1* and *mPer2* expression in the SCN and caused a behavioral phase shift. Thus, *mPer1* and *mPer2* are considered to work both in the generation of circadian rhythm and in light entrainment.

It has recently become clear that histone modification plays an important role when genes are transcribed in the nucleus and basic domains in the histone N-terminal are modified, such as by phosphorylation, acetylation, methylation, or ubiquitylation (35). In particular, the acetylation of the lysine residue in the histone N terminus by histone acetyltransferase (HAT) increases transcriptional activity, and deacetylation by histone deacetylase (HDAC) induces transcriptional repression (18, 30, 36, 46). In the study of circadian clocks, phosphorylated histone has been shown in SCN cells after a nocturnal light pulse without identifying the genes (7). More recently, rhythmic histone H3 acetylation was reported to occur in the transcription of *mPer1* and *mPer2* in the liver and heart (8, 13). However, the involvement of histone deacetylation in the circadian feedback loop and the histone acetylation-deacetylation in the light response of clock genes have not been elucidated. In the present study, we reveal that the rhythmic expression and light induction of *mPer1* and *mPer2* are regulated by histone acetylation and deacetylation.

MATERIALS AND METHODS

Plasmids, antibodies, and chemicals. cDNAs containing whole mouse *Per1* (*mPer1*), *mPer2*, *mPer3*, *mCry1*, *mCry2*, *mBMAL1*, and *mSin3B* genes were cloned into the pcDNA3 vector. The cDNAs of the *mHDAC1*, *mHDAC2*, and *mHDAC3* coding regions were obtained by reverse transcription-PCR (RT-PCR) with sequence-specific oligonucleotide primers based on published sequences. The construction of mSin3B mutants, pcDNA3-GAL4 DNA-binding domain [G4DBD(1-147)], pcDNA3-G4NRSF, and the GAL4 reporter plasmid pGL3-S10PR5GB (containing the SCG10 promoter-5xGAL4-DNA binding site) has been described previously (26). The GAL4 reporter plasmid pGL5SV containing the simian virus 40 promoter-5xGAL4-DNA binding site was provided by Y. Agata. Glutathione *S*-transferase (GST)-fused constructs were generated by the ligation of fragments of *mCry1* cDNAs in-frame into the pGEX plasmid (Pharmacia). Details of these constructions are available upon request. Anti-mSin3B (A-20), anti-HDAC1 (C-19), anti-HDAC2 (C-19), anti-mCRY1 (A-20) (Santa Cruz Biotechnology Institute), anti-cyclic AMP response element binding protein (CREB), anti-phospho-(Ser^{133})-CREB (pCREB) (New England Biolabs), anti-Flag M2 affinity gel (Kodak), and anti-Flag M2 (Sigma) antibodies were

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purchased commercially. Trichostatin A (TSA) was used as an HDAC inhibitor (Wako Pure Chemical Industries).

Reporter gene assays. NIH 3T3 cells were transfected with various plasmids with Lipofectamine Plus (Gibco-BRL). For luciferase assays, 5×10^4 cells in 24-well plates were transfected with 200 ng of luciferase reporter plasmid, 2.5 to 100 ng of effector plasmid (see the legend to Fig. 1), 50 ng of control *Renilla* luciferase vector (pRL-TK) (Promega) as an internal control for transfection efficiency, and pcDNA3 as a carrier. After 24 h, cells were treated with TSA (100 ng/ml) for an additional 24 h. Cellular extracts were prepared 48 h after transfection, and dual luciferase activities were measured with a luminometer (Lumat LB96; EG&G Berthold).

In vitro binding assays. GST pulldown assays were carried out as described previously (26). Briefly, 1 μg of GST fusion proteins bound to glutathione beads was incubated with [^{35}S]methionine-labeled in vitro-translated proteins at 4°C, washed, boiled in sodium dodecyl sulfate (SDS) loading buffer, subjected to SDS-polyacrylamide gel electrophoresis (PAGE), and visualized by autoradiography. GST fusion proteins were stained with Coomassie blue to confirm equal loading.

Immunoprecipitation and Western blotting. Subconfluent COS7 cells were transfected with 2 μg of pcDNA-mSin3B and 2 μg of Flag-tagged mCRY1 or 2 μg of pcDNA3 with Lipofectamine Plus reagent (Gibco-BRL). Immunoprecipitations were performed as described previously (26). The proteins bound to the gel beads were eluted in Laemmli loading buffer and analyzed by SDS-PAGE. Western blotting was carried out by standard procedures and visualized with an ECL-Plus chemiluminescence system (Amersham).

ChIP assay. Chromatin immunoprecipitation (ChIP) assays were performed as described previously (23). Cells or SCN tissues were fixed with formaldehyde, and cross-linked chromatin was immunoprecipitated with antibodies against acetylated histone H3, acetylated histone H4, mCRY1, CREB, or pCREB. No antibody was indicated by using a sample with a high level of acetylated histone. Immunoprecipitated DNA was analyzed by semiquantitative PCR.

The *mPer1* transcription start site region ($-202/+65$) was amplified with the following primers: 5′ *mPer1* promoter, 5′-GCTGACTGAGCGGTGTCTGA-3′, and reverse *mPer1* promoter, 5′-GAGCGCCCTCCATCCGCTTG-3′. The *mPer2* transcription start site region ($-252/+27$) region was amplified with the following primers: 5′ *mPer2* promoter, 5′-AAGTGGACGAGCCTACTCGC-3′, and reverse *mPer2* promoter, 5′-AGCGCCGCTGCCGCCGCGTC-3′. The *mPer1* cyclic AMP response element (CRE) site region ($-1762/-1550$) region was amplified with the following primers: 5′ CRE primer, 5′-CAGCTGCCTCG CCCCGCCTC-3′, and reverse CRE primer, 5′-CCCAAGCAGCCATTGCTCG C-3′. The *mPer1* E-box2 region was amplified (341 bp): 5′ E2 primer, 5′-CTTT CACAGTAGCCATTGCC-3′, and reverse E2 primer, 5′-ACAAGACACCTGT CCTGGTG-3′ from nucleotides 4462 to 4712 of AB030818. The *mPer1* E-box3 region amplified with the following primers: 5′ E3 primer, 5′-AACAGGTCTG TGTCCCAGCA-3′, and reverse E3 primer, 5′-GGACAACATGCCAGTCTG GG-3′ from nucleotides 3707 to 3963 (AB030818).

TSA experiments and RT-PCR analysis. The levels of each clock gene mRNA were determined by semiquantitative RT-PCR (28). Total cellular RNA was isolated from NIH 3T3 cells either untreated or treated with TSA (100 ng/ml), and 1-μg aliquots were reverse transcribed with reverse transcriptase (Gibco-BRL). PCRs were performed with sequence-specific primers of the clock genes (28). In most cases, PCR was performed for 18 to 30 cycles (nonsaturated conditions) with an annealing temperature of 60°C. Experiments were repeated at least twice, and reproducibility was confirmed.

Cell culture and serum shock procedures. NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with 10% fetal calf serum, 100 μg of penicillin per ml, and 100 U of streptomycin per ml. Under these conditions, the cells reached confluence after about 4 days and were kept for 2 days in medium containing 1% fetal calf serum. At the 0-h time point, the medium was changed to DMEM supplemented with 50% horse serum, and after 2 h this was replaced with serum-free DMEM (1). At the indicated times, petri dishes were washed twice with ice-cold phosphate-buffered saline and harvested in 1 ml of Trizol reagent (Gibco-BRL). At the same time, other petri dishes were fixed with formaldehyde (1% final concentration) at 37°C for 15 min. Cells were washed with ice-cold phosphate-buffered saline and lysed by adding 400 μl of lysis buffer to a cellular pellet for ChIP assays.

Animals. Male BALB/c mice at 6 weeks postpartum were maintained for 2 weeks on a 12-h light-dark cycle and then transferred to constant darkness. Animals were given food and water ad libitum. All animals were used according to the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals. The Committee for Animal Research in Kyoto Prefectural University of Medicine also approved the experiments. Livers and brains were quickly isolated from decapitated mice, frozen, and kept at −80°C until RT-PCR

and ChIP analysis. All experiments at each circadian time point were performed under dim red light.

In situ hybridization. Mice were injected at circadian time 16 (CT16) with 5 μl of TSA (500 ng/ml) in dimethyl sulfoxide or with 5 μl of dimethyl sulfoxide vehicle into the lateral ventricle through a probe inside a 23-gauge guide cannula that had been implanted with a stereotaxic apparatus (2). One hour after the injection, mice were perfused with 50 ml of ice-cold 4% paraformaldehyde in phosphate-buffered saline under deep pentobarbital anesthesia (50 mg/kg).

Coronal frozen brain sections (25 μm thick) were cut and processed for in situ hybridization as described previously (39). The antisense RNA probes for *mPer1* mRNA (736 to 1720) and *mPer2* mRNA (388 to 1898) were prepared by in vitro transcription with linearized plasmid vectors (pBluescript) with T3 or T7 RNA polymerase and a digoxigenin labeling mixture (Roche).

RESULTS

Rhythmic acetylation of histones H4 and H3 in the *mPer* promoter region precedes expression of *mPer* mRNA in fibroblast cells and the liver. The transcriptional activity of many genes is enhanced by acetylation of lysine residues in the N-terminal tails of histone proteins that are physically associated with promoter regions (18, 30, 36). We assumed that the feedback loop of the clock genes was regulated by the chromatin modification. First, we examined whether the rhythmic expression of *mPer1* in NIH 3T3 cells after serum shock correlated with the acetylation and deacetylation of histone H3 or H4 with a ChIP assay (23). Histone acetylation in the promoter region of *mPer1* fluctuated in parallel with the rhythmic expression of the *mPer1* gene (Fig. 1A). The peak of acetylated histone H4 was slightly in advance of the peak of expression of *mPer1*, suggesting that histone acetylation of the initiating region activates the transcription of the *mPer1* gene.

To investigate whether rhythmic histone acetylation also takes place in liver tissue, we carried out ChIP assays with the *mPer1* and *mPer2* promoters. Fluctuating histone H4 and H3 acetylation occurred in parallel with the rhythmic expression of the *mPer1* and *mPer2* genes. The peaks of both *mPer1* and *mPer2* mRNA occurred at CT36, but those of histone H3 and H4 acetylation were 4 and 8 h in advance, respectively (Fig. 1B and C).

When the *mPer* gene is transcribed rhythmically, histone acetylation and deacetylation are considered to occur reciprocally. If HAT and HDAC correlate to the rhythmic expression of *mPer1*, the mPER-mCRY complex which constitutes the negative limb of the circadian feedback loop may involve HDACs.

mCRY1 acts as a transcriptional corepressor and the repression is relieved by the HDAC inhibitor TSA. We next attempted to identify which protein, PER or CRY, functioned as a transcriptional corepressor. Cells were cotransfected with a luciferase reporter driven by the GAL4 sites upstream of the *SCG10* minimum promoter (a ubiquitous expression promoter) (26) (Fig. 2A) and mCRY1 or mPER1 fused to the G4DBD, and we examined whether the recruiting of these genes repressed basal transcription. G4mCRY1 repressed reporter activity in a dose-dependent manner (Fig. 2B). However, G4mPer1 had no effect on the reporter genes (Fig. 2B). Next, we examined whether transcriptional repression through mCRY1 involved histone deacetylation with TSA, a specific inhibitor of HDACs (49). Repression with mCRY1 was alleviated by TSA administration (Fig. 2C). Similar results were
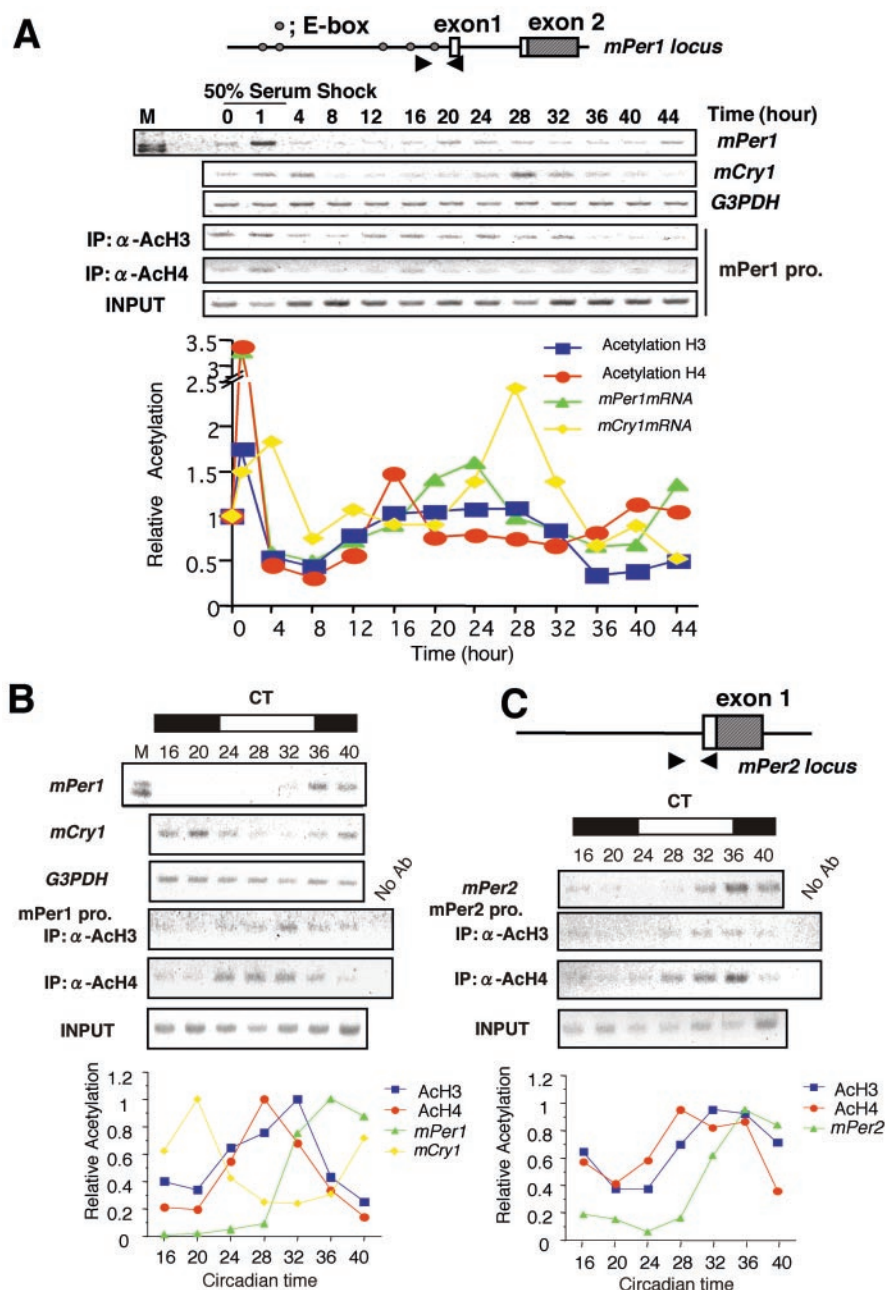
FIG. 1. Rhythmic histone acetylation of *mPer1* and *mPer2* in fibroblast cells and the liver. (A) The structure of the *mPer1* gene is illustrated, along with the region for PCR amplification in the ChIP assay. Soluble chromatin preparations from NIH 3T3 cells treated with 50% horse serum were immunoprecipitated with anti-acetylated histone H3 or H4 antibodies (α-AcH3 and α-AcH4, respectively) and analyzed by semiquantitative PCR. Aliquots of chromatin obtained before immunoprecipitation were also analyzed (input). Representative PCR results are shown in the lower three panels. The data in the upper panel were quantified with NIH Image and plotted as the change in relative acetylation and relative mRNA level. Data shown were confirmed in two independent experiments. (B) Cross-linked chromatin from liver was immunoprecipitated with anti-acetylated histone H3 or H4 antibodies and then analyzed by semiquantitative PCR with primers in the *mPer1* promoter. (C) The same ChIP assay as in panel B was performed with primers in the *mPer2* promoter. The expression of *mPer1*, *mPer2*, *mCry1*, and the control *G3PDH* gene is also shown in panels A and B.

also obtained when the promoter was changed to a simian virus 40 promoter (Fig. 2D).

mCRY1 forms a complex with mSin3 and HDAC in vitro and in vivo. Since mCRY1 binds directly to the CLOCK-BMAL1 heterodimer (17, 33), mCRY1-mediated repression may recruit another corepressor or HDACs. It is known that

many transcriptional repressors acting through HDACs bind to mSin3, a transcriptional coregulator. mSin3 proteins are known to contain four PAHs (PAHs 1 to 4), which mediate protein-protein interactions (45, 46). First, we examined whether and where mCRY1 bound mSin3 in vitro, finding that mCRY1 bound mSin3B as well as BMAL1 (a positive control)

A

Effector :　(GAL4DBD)─[mCRY1, mPER1 or NRSF]

Reporter :　──[HHHH]──────●→────[Luciferase]
　　　　　　　　　5×UAS　　SCG10 or SV40
　　　　　　　　　　　　　　　promoter
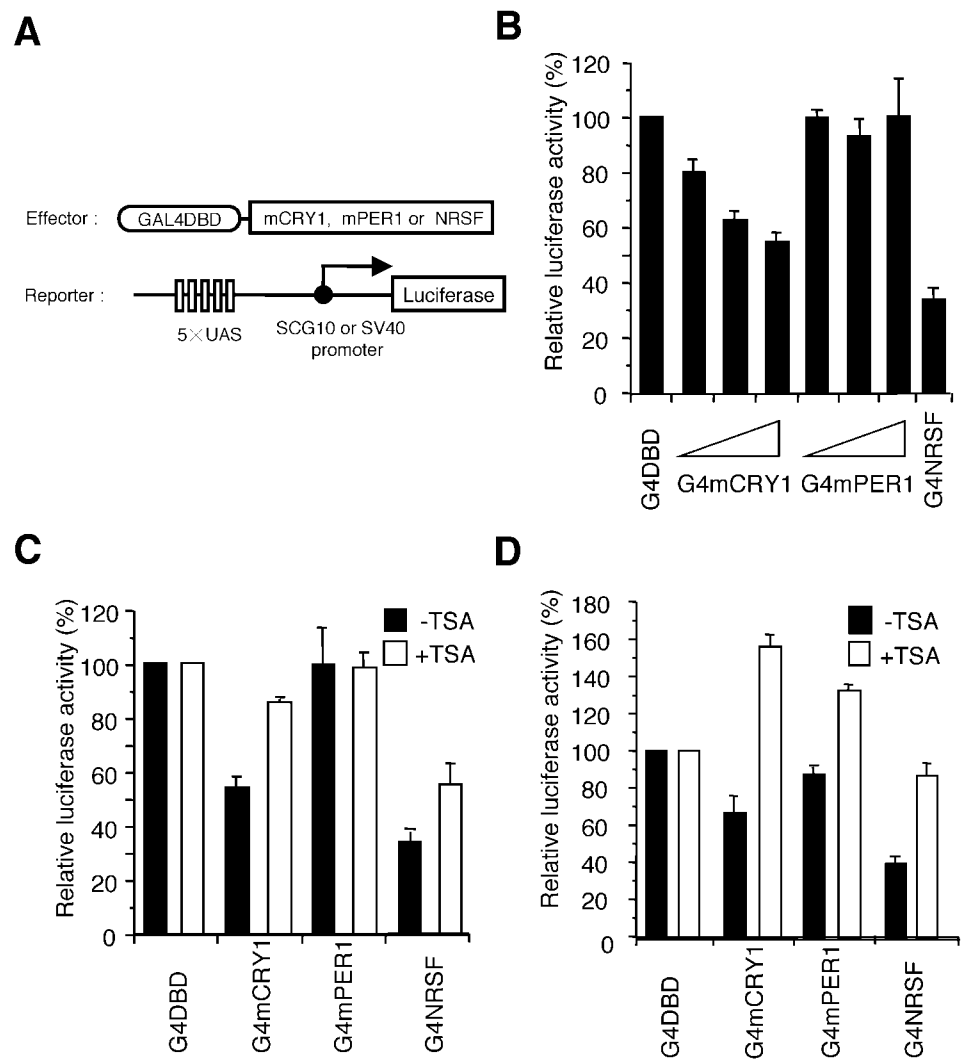
B

C

D

FIG. 2. mCRY1 represses basal transcription and the HDAC inhibitor TSA relieves this repression. (A) Schematic diagrams of the effector and reporter constructs used in the transient transfection assays. (B) NIH 3T3 cells were transfected with reporter constructs pGL-S10PR5GB (200 ng) and pRL-TK (50 ng) as internal controls as well as increasing amounts of plasmids expressing G4DBD-fused mCRY1 (G4mCRY1) (2.5, 25, and 100 ng), G4DBD-fused mPER1 (G4mPER1) (2.5, 25, and 100 ng), fused NRSF (G4NRSF) (100 ng) as a positive control, or G4DBD alone (100 ng) as a negative control. (C) NIH 3T3 cells were transfected with the same reporter as in panel A and equal amounts of G4DBD fused to each protein. After 24 h, the cells were treated with TSA (100 ng/ml) for an additional 24 h. (D) An experiment similar to that in panel C with a reporter containing the simian virus 40 promoter instead. The luciferase activities of all experiments are expressed as the mean ± standard error of the mean of at least three independent experiments performed in duplicate.

(17, 33) (Fig. 3A). We also demonstrated that the minimum binding region was PAH 1 and 2 (amino acids 1 to 299) of mSin3B (Fig. 3A).

We then investigated whether mCRY1 interacted with HDAC1, HDAC2, or HDAC3 in vitro and found that mCRY1 bound to HDAC1 and HDAC2, while the binding affinity for HDAC3 was weaker (Fig. 3B). We also tested for a possible in vivo interaction between mCRY1 and the mSin3B-corepressor complex involving HDAC1 and HDAC2 with an immunoprecipitation assay. mCRY1 bound to overexpressed mSin3B and endogenous Sin3B (upper band, Fig. 3C left), and these immunoprecipitated complexes included HDAC1 and HDAC2 (Fig. 3C). We then confirmed the rhythmic binding to the second E-box (Fig. 3D) and the third E-box (data not shown)

in the proximal promoter of *mPer1* in NIH 3T3 cells after serum shock. These results indicate that mCRY1 recruits the mSin3-HDAC1/2 complex. We also detected an obvious interaction between mCRY1 and mSin3B at 28 h, when *mPer1* expression is declining, and not at 20 h, when *mPer1* expression is increasing after serum shock (Fig. 1A and 3E).

TSA induces endogenous *mPer1* and *mPer2* mRNA and shifts the *mPer1* rhythmic phase in serum-shocked fibroblast cells. To determine whether the transcriptional repression of *mPer1* involved histone deacetylation in NIH 3T3 cells, we examined the effect of TSA on the expression of endogenous clock genes, including *mPer1*. Endogenous transcripts of *mPer1* increased in TSA-treated NIH 3T3 cells after 1 h, while no effect on the expression of glyceraldehhyde-3-phosphate dehy-
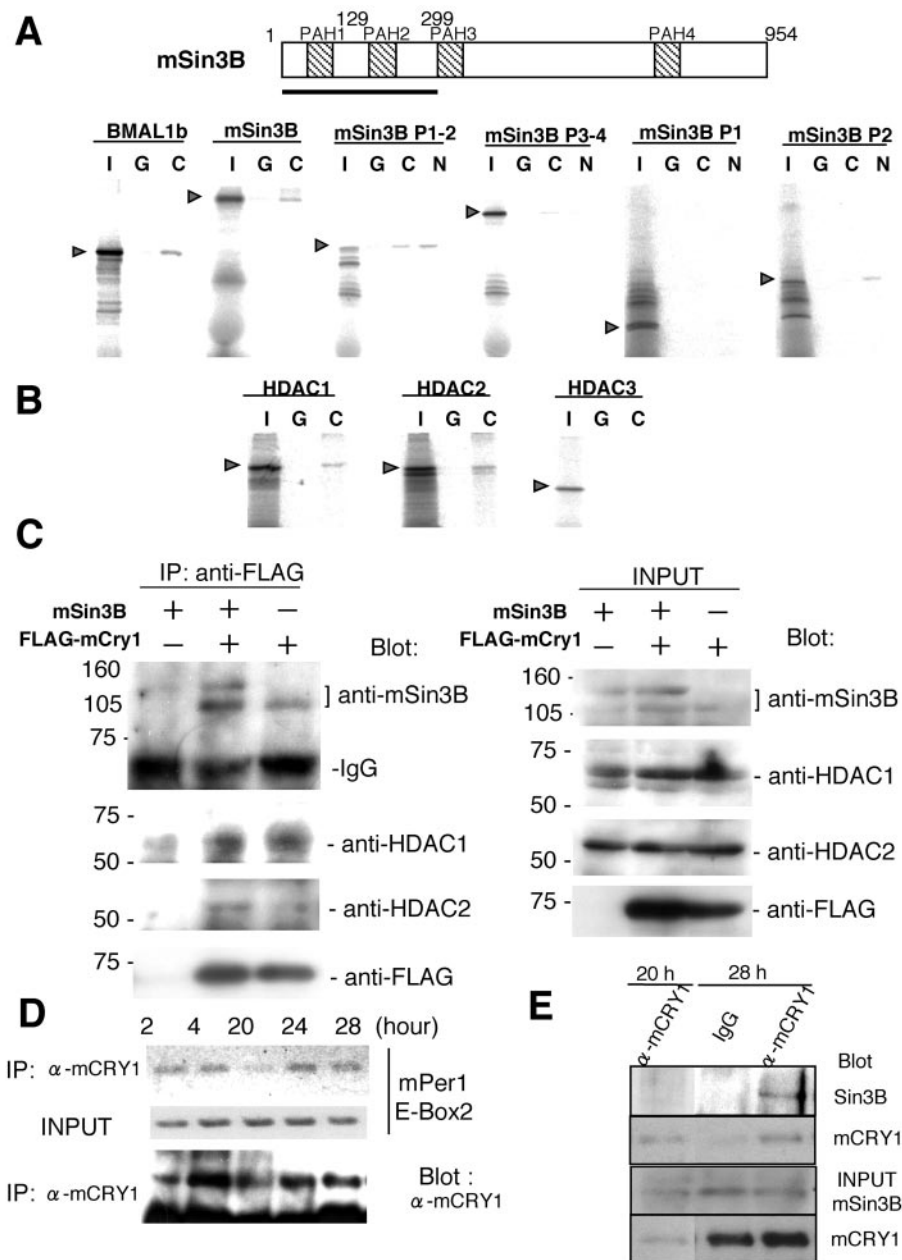
FIG. 3. mCRY1 interacts with mSin3B, HDAC1, and HDAC2 in vitro and in vivo. (A) In vitro-translated ^{35}S-labeled BMAL1, full-length mSin3B, and various deletion mutants of mSin3B were incubated with an immobilized fusion protein containing GST and mCRY1 or NRSF (amino acids 1 to 153) (N). Input protein (I) (20% of total), protein bound with GSTmCRY1 (C), protein bound by GSTNRSF-N (N), and protein bound with GST alone (G) were analyzed by SDS-PAGE. BMAL1 and GST-NRSF (N) were positive controls. A schematic representation of the mSin3B interaction domain with mCRY1 is also shown. (B) In vitro-translated ^{35}S-labeled mHDAC1, mHDAC2, and mHDAC3 were incubated with an immobilized fusion protein containing GST and mCRY1. The input (I) shown is 20% of the amount used in each incubation. (C) COS7 cells were transfected with expression plasmids encoding Flag-tagged *mCry1* (Flag-*mCry1*) and *mSin3B*, and cell extracts were immunoprecipitated (IP) with anti-Flag M2 antibodies, followed by immunoblotting analyses with antibodies against mSin3B, HDAC1, HDAC2, and Flag. Input (5% cell extracts) was applied to ascertain the positions of the blotted proteins. (D) mCRY1 is bound to the *mPer1* E-box after serum shock in NIH 3T3 cells. After serum shock, NIH 3T3 cells were cross-linked and immunoprecipitated with antibody against mCRY1. Precipitated DNA was then amplified with primers flanking the second mPer1 E-box with a semiquantitative PCR method. Input represents signals derived with input chromatin as template. Immune complexes were Western blotted and probed for mCRY1. (E) mCRY1 association with mSin3B in serum-induced NIH 3T3 cells. Cross-linked extracts were immunoprecipitated with an antibody against mCRY1 or with immunoglobulin G at 20 and 28 h after serum shock in NIH 3T3 cells. The immune complexes were Western blotted and probed for mCRY1 and mSin3B. The input shown is 5% of the total extracts.
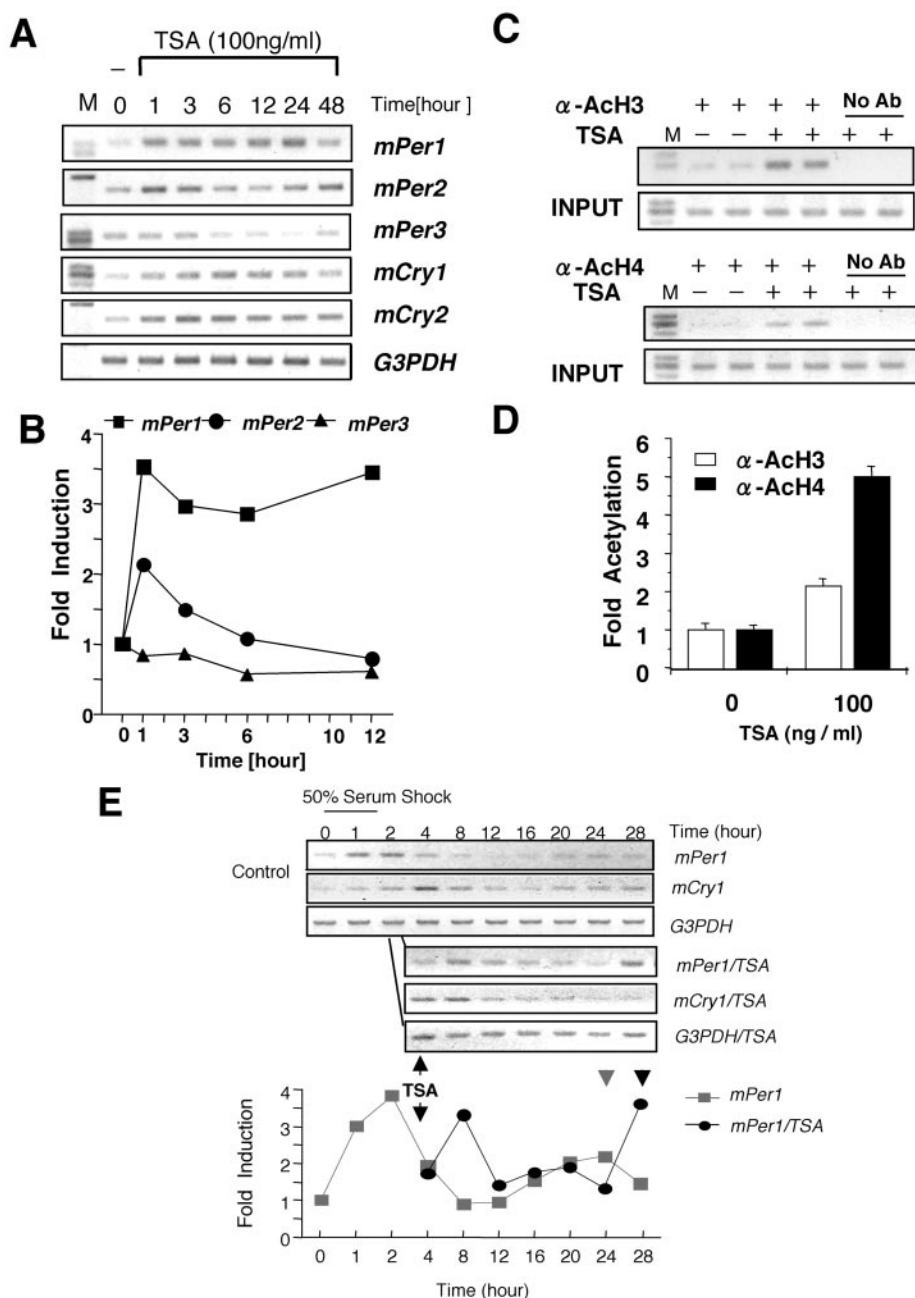
FIG. 4. TSA induces endogenous *mPer1* gene expression and acetylation of histones at the *mPer1* promoter. (A) NIH 3T3 cells were incubated with TSA for the duration indicated, and expression levels of various mRNAs were determined by semiquantitative RT-PCR (26, 28). Five clock genes, *mPer1*, *mPer2*, *mPer3*, *mCry1*, and *mCry2*, as well as a constitutively expressed control gene, *G3PDH*, are shown. (B) The data in panel A were quantified with NIH Image and are shown graphically. (C) Soluble chromatin preparations from NIH 3T3 cells treated with TSA for 1 h were immunoprecipitated with anti-acetylated histone H3 or H4 antibodies (α-AcH3 and α-AcH4, respectively). ChIP samples were analyzed by semiquantitative PCR with primers from the promoter (transcription start site region) of the *mPer1* gene. (D) Semiquantitative PCRs were carried out three times per experiment shown in panel C and are plotted as the average change in acetylation (mean ± standard error of the mean). (E) TSA can shift the phase of the rhythmic expression of *mPer1* and *mCry1* in fibroblast cells. NIH 3T3 cells at 4 h after serum shock were incubated with TSA or vehicle (control) for 1 h, and then the expression levels of *mPer1* and *mCry1* mRNA were determined by RT-PCR. The lower graph shows the data in the upper panel after quantification with NIH Image.

drogenase (*G3PDH*) was observed (Fig. 4A). Transcripts of *mPer2*, *mCry1*, and *mCry2* were also upregulated by TSA, but that of *mPer3* was unaffected (Fig. 4A and B).

We then examined whether the increase in *mPer1* gene ex-

pression induced by TSA in NIH 3T3 cells was accompanied by a change in histone (H3 and H4) acetylation at the *mPer1* promoter with a ChIP assay (Fig. 4C). Treatment with TSA for 1 h dramatically increased the acetylated form of histone H4
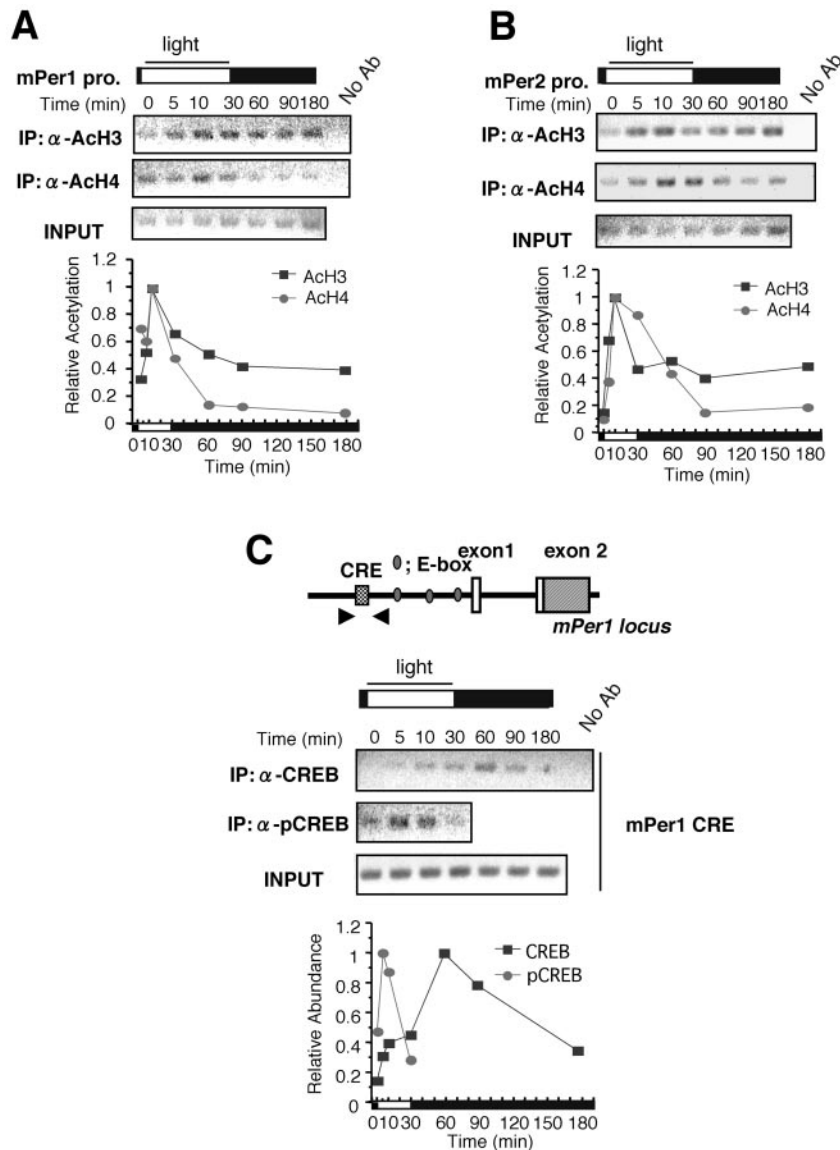
FIG. 5. Light induction of histone H3 and H4 acetylation in the *mPer1* and *mPer2* promoters and binding of CREB and pCREB to the CRE site on the *mPer1* locus in the SCN. Mice were exposed to a 600-lux light pulse for 30 min at CT16. Six brains were collected at each time point, 0, 5, 10, 30, 60, 90, and 180 min after the light pulse, and processed for the ChIP assay. Cross-linked chromatin from SCNs was immunoprecipitated with anti-acetylated histone H3 or H4, anti-CREB, and anti-pCREB antibodies and then analyzed by semiquantitative PCR with primers for the *mPer1* (A), *mPer2* (B), or *mPer1* CRE site (C) promoters. Aliquots of chromatin obtained before immunoprecipitation were also analyzed (input).

associated with the endogenous *mPer1* promoter region (Fig. 4C and D), and moderately increased the acetylated histone H3 (Fig. 4C and D). Next, we investigated whether the phase of *mPer1* rhythmic expression was changed by TSA. TSA treatment at 4 h, when *mPer1* was declining after serum shock in NIH 3T3 cells, rapidly reincreased *mPer1* expression and caused a phase shift for 4 h (Fig. 4E). TSA treatment at 4 h also shifted the expression of *mCry1* (Fig. 4E). However, the level of constitutively expressed *G3PDH* mRNA was unaffected. *mPer1* expression did not change with TSA treatment when its level was augmented at 1 or 2 h after serum shock (data not shown).

Light induction of *mPer1* and *mPer2* mRNA was associated with histone H3 and H4 acetylation at their promoters, and light increased the binding of pCREB and CREB to the CRE site on the mPer1 locus in the SCN. *mPer1* and *mPer2* mRNAs are rapidly induced in the SCN by light exposure during nighttime (34, 38). We examined histone acetylation at the promoters of *mPer1* and *mPer2* induced by light with a ChIP assay; finding that it increased after the light stimulus, with a peak at 10 min. The level of histone H4 acetylation then decreased rapidly, whereas histone H3 acetylation did not decline but remained at a relatively higher level (Fig. 5A and B). No band was detected in the control containing no antibody.
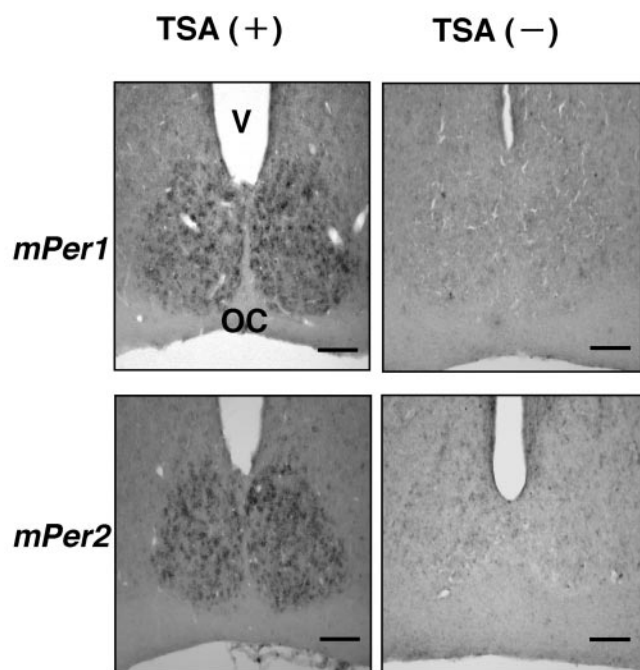
FIG. 6. mPer1 and mPer2 induction by TSA in the SCN. TSA or vehicle was administered at CT16. By in situ hybridization with digoxigenin-labeled probes, mPer1 and mPer2 mRNAs were demonstrated in the whole SCN 1 h after TSA treatment. OC, optic chiasm; V, third ventricle. Bars, 100 μm.

Although CREB and pCREB after light stimuli have been considered important for time resetting (10, 15), there have been no reports which demonstrated the direct binding of these with the CRE site. We attempted ChIP assays with anti-CREB and anti-pCREB antibodies at the CRE close to the promoter of mPer1. The level of CREB binding to the CRE site reached a peak at 60 min after the light stimulus (Fig. 5C). On the other hand, the level of pCREB reached a peak at 5 min after the light stimulus and rapidly returned to the basal level at 30 min. CBP and p300, which are HATs, are known to bind to pCREB (9, 16, 27). These results suggest that histone acetylation of the mPer1 promoter after light exposure correlates with the phosphorylation of CREB.

TSA induces mPer1 and mPer2 expression in the SCN. If histone acetylation and deacetylation are important for the expression of mPer1 and mPer2 by light stimulus, TSA administration into the SCN may induce these genes as well. One hour after TSA was injected into the lateral ventricle at CT16, mPer1 and mPer2 mRNAs were induced in neurons of the SCN (Fig. 6). These results suggest that histone acetylation and deacetylation are involved in the regulation of mPer1 and mPer2 expression in vivo and that inhibitors of HDACs may phase shift the circadian rhythm.

DISCUSSION

Molecular mechanism of rhythmic mPer1 and mPer2 expression. In this study, we demonstrated that chromatin modification through histone acetylation and deacetylation participated in regulation of oscillating mPer1 transcription in fibroblast cells and liver. The negative regulator, mCRY1, repressed transcription and bound to the HDAC complex. Furthermore, an HDAC inhibitor, TSA, activated mPer1 and mPer2 transcription and accelerated the acetylation of histones H3 and H4 at the mPer1 promoter. From these results, we propose that the circadian feedback loop of mPer1 transcription is regulated by four processes. The first process is transcriptional activation by recruitment of the CLOCK-BMAL1 complex, including HATs and CBP/p300 (13, 37), onto the E-box in the promoter. The second is that the translated mPER forms a complex with mCRY, which is phosphorylated by CKIε/δ in the cytoplasm and then enters the nucleus. The third is the inhibiting process by the phosphorylated mCRY-mPER complex with HDACs that bind to the CLOCK-BMAL heterodimer. The final process is relief of the repressive mechanism; it is unknown whether the fourth process is caused by export of a negative regulator (42), protein degradation, or participation of another transcriptional factor. Together, these processes compose an approximately 24-h cycle of clock gene oscillation. The way in which histone deacetylation is inhibited seems to be an important point for the activation of mPer1 transcription, because transcription is immediately activated only by inhibition of HDAC activity with TSA.

A recent study revealed that histone H3 acetylation on the mPer1 and mPer2 promoters exhibited synchronous rhythm in mPer1 expression in liver (13). The present results demonstrated that not only histone H3 but also H4 was acetylated; H4 was acetylated first, then that of H3 increased, and then they fluctuated preceding the rhythm of mPer1 and mPer2 mRNA. Echegaray et al. reported that the peak of H3 acetylation coincided with the peak of mPer1 expression (13). However, Curtis et al. recently showed that the peak of H3 acetylation in the human (h) Per1 promoter comes 6 h before that of the hPer1 mRNA level in the heart and in serum-shocked HeLa cells (8). They also showed rhythmic H4 acetylation in HeLa cells. The reason for the 4-h difference in the H3 acetylation rhythm in the liver between our study and the earlier report (13) is uncertain but could be in part due to the difference in the method for the ChIP assay; in which we used sonicated cell lysate, while Echegaray et al. used nuclear extracts. In general, gene expression is regulated by a complex of HATs that specifically recognize the lysine residue of the histone tail (12, 20). Different states of chromatin modification in each clock gene or the involvement of other transcription factors might have caused the time lag in clock gene expression induced by TSA treatment of fibroblast cells in this study.

The behavior of $Cry1^{-/-}$ $Cry2^{-/-}$ double mutant mice was reported to be arrhythmic under constant darkness, with constantly elevated mPer1 expression, and no rhythmicity was detected in either 12-h light/12-h dark or constant darkness (29, 44). In these mice, we suspect that mPer1 expression was maintained at a high level, as mCRY1 and mCRY2 could not recruit the mSin3-HDAC complex into the CLOCK-BMAL1 complex, which is also known to be associated with the HATs CBP/p300 (13, 37), in activating transcription. The fact that expression of mPer in $BMAL1^{-/-}$ mutant mice was at a constant low level (6) might be due to the unsuccessful recruitment of HATs into the E-box of the mPer locus. Thus, the extent of histone acetylation on the mPer1 promoter is thought to be

elevated in CRY double mutant mice and reduced in BMAL1 mutant mice.

Regulation of the light response in the SCN. The remarkable increase in CREB phosphorylation observed in the SCN and the CREB-dependent signaling are considered to be important for *mPer1* activation after light exposure in subjective night (10, 15, 40, 41). It is known that pCREB binds to a transcriptional coactivator and HATs such as CBP and p300 and regulates gene expression through chromatin remodeling and acetylation of other transcriptional factors (9, 16, 27). We demonstrated that CREB or pCREB bound directly to the CRE site close to the *mPer1* promoter. Histone acetylation on the *mPer1* promoter may be caused by recruitment of the transcriptional complex to the CRE site via CREB-CBP. CRE-binding pCREB declined earlier than the expression of *mPer1* mRNA in the SCN, which showed a peak at about 1 h after the light exposure (34). In contrast, the state of acetylated histones binding to the promoter of *mPer1* seems to be linked with the expression of this gene. Thus, after light exposure, pCREB binds to the CRE first, then acetylation of histones H3 and H4 occurs, and finally *mPer1* seems to be transcribed in the SCN. Further, the fact that TSA administration at CT16 induced *mPer1* and *mPer2* expression in the SCN as well as that induced by the light response means that the nadir in circadian expression of the *mPer* genes is related to the activation of HDACs, suggesting that the mechanism of histone acetylation and deacetylation is also important for light-induced expression of these genes.

Only 2% of endogenous genes in cells are considered to be affected by HDAC inhibitors (32). Some cancer-related genes have been shown to be affected by HDAC inhibitors, and studies on the development of anticancer drugs have been advanced with their actions (25, 43). From the present results, HDAC inhibitors may also be possible target drugs for circadian phase shift.

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